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In vitro investigations on the effects of semi-synthetic, sulphated carbohydrates on the immune status of cultured common carp (*Cyprinus carpio*) leucocytes.

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Key words: Immunostimulants; Beta-glucan; Carbohydrates modification; Immune responses; Innate immunity; Cytokines; *Cyprinus carpio*.

Highlights

- Modification to methyl hydroxyethyl cellulose produced a novel carbohydrate (MHCS).
- Modified glucan (MHCS) exhibited consistent biological activities.
- MHCS induced strong respiratory burst in leucocytes without impacting viability.
- MHCS induce the expression of inflammation-related genes in carp immune cells.
- MHCS enhanced the effect of Poly I:C on carp leucocytes.

Abstract

The rapid emergence of drug resistance, unfavourable immunosuppression and mounting evidence to suggest the deleterious accumulation of drug breakdown residues within animal tissues has driven a strong desire to move away from these current methods of disease control. Some natural products such as β-glucan, which are extracted from, for example, plants and fungi, are able to modulate the immune system and increase protection against diseases. However, these products are heterogeneous and their effects can be variable thus limiting their applicability and reliability. Carbohydrates were modified via chemical sulphation and these semi-synthetic, sulphated carbohydrates analysed for their immunological activity utilising carp pronephric cells and a carp leucocyte cell line (CLC). A sulphated β(1,4)-glucan, methyl hydroxyethyl cellulose sulphate (MHCS), demonstrated a stimulatory effect on fish immune cells. MHCS induced a range of bioactive effects in carp leucocyte cells whilst not affecting cell viability when cells were exposed for 24h at concentrations of 1-150 $\mu g ml^{-1}$. MHCS stimulated the innate immune system where a significant increase in respiratory burst activity was observed at concentrations 25-250 $\mu g ml^{-1}$ in comparison to control (sterile water), cellulose ether, MacroGard® and zymosan. Also, under in mock bacterial and viral infection conditions i.e. Lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (Poly(I:C)), MHCS enhanced the immune responses of pronephric cells by stimulating the respiratory burst activity at concentrations 50 and 150 $\mu g ml^{-1}$. MHCS also enhanced the expression of cytokines including interleukin 1 beta (IL1β),

tumor necrosis factor alpha 1 and 2 (TNFα 1,2), interferons alpha 2 (IFN α2) and inducible nitric oxide synthase (iNOS) in carp pronephric cells. It is proposed that this new semi-synthetic carbohydrate is a potential candidate for the development of a new generation of immunostimulants and adjuvants for use in vaccination strategies in aquaculture.

1. Introduction

Aquaculture is a rapidly developing sector in animal production; however infectious diseases remain a major obstacle to the expansion of this industry. The widespread use of antibiotics and chemotherapeutics has conventionally been deployed to ameliorate undesirable infections. The rapid emergence of drug resistance, unfavourable immunosuppression and mounting evidence to suggest the deleterious accumulation of drug breakdown residues within animal tissues such as Microcystin-LR in Yellow Perch (*Perca Flavescens*) (Dyble et al., 2011), has driven a strong desire to move away from these current methods of disease control, thus alleviating their associated negative environmental and potential health-associated impacts (Anderson, 1992). Routine vaccination, used to strengthen the immune system in the fish and protect against infection, has emerged as an effective and economically viable means of disease control although to-date, many infectious diseases of worldwide importance are not currently preventable by vaccination programmes.

Recent research has concentrated on the development of natural disease control strategies, which bolster the immune system of the fish through the administration of immunomodulatory compounds (Maudling, 2006). Such immunomodulators are able to regulate the immune system through their innate ability to stimulate and/or suppress various, distinct components within the immune system of fish (Zapata et al., 1997). An example of an immunomodulatory agent that has found widespread use in aquaculture is the carbohydrate immunostimulant, β(1,3 ± 1,6)-glucan. These naturally derived carbohydrates act by enhancing both the innate and adaptive immune system. Administration is normally carried out through injection, although the use of less invasive immersion bath technologies and/or formulation within fish feeds are the preferred methods of deployment (Herman, 1970).

Beta-glucan, a polysaccharide composed of repeating β-D-glucose monomers linked by 1,3 and 1,6 glycosidic bonds, is obtained from the cell wall of many microorganisms, cereals, fungi, seaweed and algae. The most frequent sources are baker's and brewer's yeasts *Saccharomyces cerevisiae* (Novak and Vetvicka, 2008, Petravić-tominac et al., 2010), which have been investigated in both laboratory and clinical studies.

Natural β-glucans exhibit a variety of immune-related activities that are dependent on underlying composition and fine structure, molecular weight, linkage type and branching pattern; these also dictate the varying solubility of this class of molecules (Li et al., 2013). This structural complexity and their inherent batch-to-batch variability leads to difficulties in predicting the immunoactivity profile of β-glucans, which are also capable of provoking undesirable side-effects when such heterogeneous immunomodulants are utilised as feed supplements or adjuvants. Both the Centre for Veterinary Medicine (CVM) and the Food and Drug Administration (FDA) in the United States have implemented several requirements, which must be adhered to for regulatory approval of aquaculture feeds and drugs. These requirements includes general examinations i.e. the determination of physicochemical parameters (e.g. pH), homogeneity, the presence of foreign particles, or microbial contamination (e.g. *Salmonella*, *Coliform* and *Vibrio*) and the detection of heavy metal contaminants (e.g. lead, cadmium and mercury). And special examination parameters include both qualitative and quantitative testing to elucidate an in-depth structural analysis of the products. Therefore, it is desirable that any immunomodulatory agent, which is to be formulated with animal feed, or deployed as a drug, has an identifiable structure that can be reproduced in a facile manner, free from batch-to-batch variation.

Natural polysaccharides can be augmented by sulphation, thereby producing physically and chemically modified polymers to assist the development of new biomaterials. The

aforementioned modifications may bestow important therapeutic and biological activities, examples being the modulation of coagulation by sulphated oat β-glucan (Chang et al., 2006), the antitumor potential of sulphated α-(1-3)-D-glucan obtained from the fruiting bodies of *Ganoderma lucidum* (Zhang et al., 2000) and microbial invasion blocking, e.g. HIV with sulphated curdlans (Yoshida et al., 1995), or sulphated *Konjac glucomannan* (Bo et al., 2013). These bioactive carbohydrates are dependent upon the presence of sulphate groups that play an important role in a variety of regulatory and modulatory processes, combined with binding and recognition events between specific carbohydrate structures (negatively charged sulphated group) and their protein partners (usually positively charged peptide sequences). Binding is affected by the presence of suitable polar groups, the degree of sulphation, associated cations, molecular weight and the chain conformation of the polysaccharides (Toida et al., 2003, Bo et al., 2013).

The mechanism of action of sulphated carbohydrates in carp is currently unknown but, it seems likely that at least in part, it is the result of the ability of sulphated carbohydrates to mimic the endogenous glycosaminoglycan (GAG) polysaccharides. The GAGs are naturally occurring, sulphated carbohydrates that are well conserved through evolution, with GAG species identified in bacteria, fish, reptiles, molluscs, arachnids, insects and mammals (Volpi, 2005). Numerous, distinct roles for the GAG class of polysaccharides have been elucidated in recognition, binding, regulation and modulation of many proteins, including those involved in the hosts immune system (Skidmore et al., 2008, Rudd et al., 2010a). Indeed, growing evidence suggests that the physiological role of heparin, a pharmaceutical anticoagulant, is most likely to be in an immunomodulatory capacity and not the antithrombotic capacity for which it is best known. Previous work by the authors has demonstrated that semi-synthetic, sulphated carbohydrates can mimic the biological activities of the glycosaminoglycan (GAG) class of naturally occurring, sulphated carbohydrates (Rudd et al., 2010b). This study has pre-screened an extant library of sulphated carbohydrates, acting as GAG analogues, which have been shown previously to possess favourable bioactivity in biological systems known to be modulated by GAGs (Rudd et al., 2010b, Boyle et al., 2017, Skidmore et al., 2017). The constituent sulphated glycans of the library resource cover a wide spread of chemically diverse sequence-space (including chemically sulphated β-glucans), and this study as identified a sulphated β(1,4)-glucan, methyl hydroxyethyl cellulose sulphate (MHCS), as a potential candidate for favourable immunomodulation in aquaculture with future potential as an adjuvant. Furthermore, the negligible cytotoxicity of this carbohydrate-based candidate has been demonstrated, along with its favourable immunostimulatory potential within the inflammatory response of relevant fish cells.

2. Materials and Methods

2.1 Preparation of MacroGard®, zymosan and cellulose ether immunostimulants

Concentrations of MacroGard® (Biorigin; a bakers' yeast extract containing 60% of β-1,3:β-1,6 glucan), zymosan (Sigma, Z4250; a β-1,3 linked glucan) and cellulose ether (Tylose) (Sigma 93802; a linear, β-,1,4 linked glucan) were prepared as described by Vera-Jimenez et al. (2013). Owing to the innate insolubilities of the parental material appropriate concentrations of the aforementioned carbohydrates were made-up in sterile-filtered water (Sigma, W3500) and sonicated twice for 30 s (Sonics Vibra-cell, power setting 6). To ensure sterility, the stock solution was heated to 80ºC for 20 min prior to incubation at 19 ± 1ºC.

2.2 Preparation of pathogen associated molecular patterns (PAMPs)

LPS from *E.coli* 0111:B4 strain (Invitrogen) and Poly(I:C) (a synthetic analogue of dsRNA; Invitrogen), were prepared as per the manufacturer's instructions and diluted to the required concentration with sterile-filtered water.

2.3 Preparation of semi-synthetic sulphated carbohydrate based immunostimulants

Semi-synthetic carbohydrates were sulphated by a modified version of the chlorosulfonic acid (CSA) sulphation protocol as described by Yoshida et al. (1995), which modifies amenable hydroxyls. Briefly, the powdered precursor carbohydrates (0.5 g) were dissolved in ice-cooled 5 ml dimethylformamide (Sigma), 10 ml pyridine (VWR) and 1 ml chlorosulfonic acid (Sigma). The mixtures were heated to 95 ℃ for 2 h, cooled over ice and slowly neutralized with sodium hydroxide (50% w/v; VWR). Ethanol precipitations were performed in saturated sodium acetate (Sigma), overnight at 4 ℃. Precipitates were dissolved in deionized water and dialysed (Mw cut off > 7 kDa; VWR) for 72 h against HPLC grade water (VWR). The sulphated carbohydrates were lyophilised and resuspended in sterile filtered water at appropriate concentrations prior to use. Confirmation of sulphation and the degree of sulphation of bioactive saccharides was achieved using sodium rhodizonate, based on the method described by Terho and Hartiala (1971) [supplementary data, Figure 1]. Further evidence of precursor modification was obtained using Attenuated Total Reflection Fourier Transform Infrared (FTIR-ATR) spectroscopy [supplementary data, Figure 2].

2.4 *In vitro* Carp leucocytes cell line culture.

Carp leucocytes cell lines (CLCs) is a permanent cell line established from peripheral blood mononuclear cells obtained from a normal, non-leukemic, non-virally infected common carp. CLC morphology characterise with an epithelial like shape and exhibiting functions similar to monocytes and macrophages including adherence to plastic and phagocytosis of iron particles (Faisal and Ahne, 1990). CLC exhibited respiratory burst activity after stimulation with Phytohaemagglutinin (PHA) and LPS and this was similar to head kidney macrophages responses (Koumans-van Diepen et al., 1994). CLC suitability for studies on macrophage activation, and as *in vitro* model to study the immune responses of fish was concluded in both studies by Weyts et al. (1997) and Vidal et al. (2009). In addition, the CLC stimulate leukocyte proliferation by producing interleukin-1like factors (Weyts et al., 1997). The CLC line were kindly provided by Wageningen University, The Netherlands and were grown at 27°C and 5% CO_2 in L-glutamine free RPMI (Sigma) modified with 5%

(v/v) foetal bovine serum (Sigma), 2.5% (v/v) heat-inactivated pooled carp serum, 50 U/ml penicillin-G, and 50 $mgml^{-1}$ streptomycin (Sigma, P4458); this modified medium referred to hereafter as CLC $RPMI^{+}$. Cultures were split (1:3 v/v) when reaching 80% confluence; culture medium was replenished every 3 days. The trypan blue exclusion assay (Howard and Pesch, 1968, Hauton and Smith, 2004) was used to determine cell viability and only cell suspensions with at least 95% viability were used for experimentation.

2.5 Preparation of pronephric cell suspension

Common carp, *Cyprinus carpio* (Fair Fisheries, Shropshire, UK), were maintained in black plastic tanks with recirculating water at 15 °C and pH 7, and kept on a 12 h:12 h, light: dark cycle. Approximately 25 fish were kept in each tank and were fed daily on commercial pelleted feed that lacked an immunostimulant additive (Tetra GmbH, Germany). The pronephros was removed from 5 carps (89.6 ± 12.4 g), which had been sacrificed previously by a lethal dose (~ 0.2% v/v) of 2-phenoxyethanol (Sigma). Blood was collected from the caudal vein before dissection. The isolated pronephros was placed in modified RPMI medium on ice, under sterile conditions and a cell suspension was prepared using a modification of the procedure described by Kemenade et al. (1994). Briefly, pronephros tissue was disrupted gently through a sterile cell strainer with 100 μm pore diameter (BD Falcon) in 1 ml of modified RPMI medium that comprised RPMI supplemented with 0.3 gL^{-1} L-glutamine (Sigma), 0.5% (v/v) sterile water, 0.05% (v/v) heat-inactivated pooled carp serum, penicillin (50 U/ml), and streptomycin (50 μgml^{-1}) (Sigma); this modified medium referred to hereafter as $RPMI^{+}$. A non-continuous Percoll gradient (Sigma) was used to isolate leucocytes, which were collected at the interphase between densities 1.02 gml^{-1} and 1.08 gml^{-1}, washed three times with $RPMI^{+}$ and centrifuged at 4℃ (800 g; 10 min).

2.6 MTT cell proliferation assay

Potential toxic effects on cell proliferation of administered semisynthetic, sulphated carbohydrates were determined utilising the tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) assay and compared to other immunostimulants. Briefly, a serial dilution of the test carbohydrate (1-150 μgml^{-1}) was prepared and added to CLCs (2×10^4 cells in 100 μl per well) in a 96 multiwell plate (Sarstedt). A negative control comprising 5 μl sterile water and positive controls comprising 50 μgml^{-1} of MacroGard® and zymosan were also included. After 24 h the MTT assay was performed by adding 10 μl per well of the MTT solution (5 mg of MTT; Sigma) dissolved in 1 ml of PBS (Life technologies). The plate was then incubated for 4 h at 27 ˚C with 5% CO_2, the supernatant discarded, the cells solubilised with 100 μl of dimethyl sulfoxide (Fisher) and the relative levels of proliferation measured indirectly by spectrophotometry at a λ_{abs} of 540 nm (Ferrari et al., 1990).

2.7 Trypan blue cell viable cell count assay

Trypan blue cell viability assays were conducted with and without the addition of semisynthetic, sulphated carbohydrate addition and other relevant immunostimulants. CLC lines (2×10^5 cells in 1 ml of CLC $RPMI^{+}$ medium) were distributed in 24 wells plates and stimulated by adding 15 μl per well of carbohydrate sample at 1, 2.5, 50, 150 μgml^{-1}. A negative control comprising 15 μl per well of sterile water and a positive control comprising 15 μl per well of MacroGard® at a concentration of 50 μgml^{-1} were also included. After 24 h

incubation at 27℃ and 5% CO_2, the culture medium was aspirated, and the cells washed once with pre-warmed CLC RPMI$^+$ medium. Cells were then detached by adding 250 µl per well of 0.25x Trypsin-EDTA (Sigma) for 1 min, washed 3 times with 500 µl of CLC RPMI$^+$ medium, centrifuged at 750 *g* for 5 min at 19 ± 1℃ and the supernatant discarded. The cell pellet was re-suspended in 500 µl of fresh CLC RPMI$^+$ and viable cells were determined using trypan blue solution (0.4% w/v).

2.8 Respiratory burst activity screen

The NBT assay was performed as described by Vera-Jimenez et al. (2013) to determine respiratory burst activity. Briefly, CLCs were placed in CLC RPMI$^+$ with the exception that the RPMI medium used was free of phenol red (Sigma). A cell monolayer was formed at the bottom of the flat 96 multiwell plate (Sarstedt), which were incubated at 27C˚ in 5% CO_2 for 2-3 h. The supernatants were discarded, the cells washed with phenol red free Hank's balanced salt solution (HBSS) (Sigma) and 160 µl of CLC RPMI$^+$ containing NBT at 1 mgml^{-1} (Sigma) was added to each well. The respiratory burst activity was induced by adding 5 µl of increasing concentrations of test solutions containing either MacroGard® (1-150 µgml^{-1}), zymozan (1-150 µgml^{-1}), methyl hydroxyethyl cellulose (2.5-250 µgml^{-1}) or the sulphated derivative of the latter (MHCS; 1-250 µgml^{-1}). Poly(I:C) (100 µgml^{-1}) and LPS (50 µgml^{-1}) were also assayed as non-carbohydrate controls. After incubation at 27˚C in 5% CO_2, the supernatants were decanted, the cells fixed with ice cooled methanol (100 µl, 3 min) and the plates left to air dry. The membranes of the phagocytic cells were solubilised with 120 µl KOH (2 M) and 140 µl of DMSO added to solubilise the blue formazan. The reduction of NBT was measured spectrophotometrically at λ_{abs} of 620 nm.

2.9 Carbohydrate based modulation of immune-associated gene expression

Pronephros cells prepared from 3 fish individually at 4×10^6 cells per well, in 2 ml were cultured in 6 well plates (Sigma) and exposed to 60 µl of either MacroGard® (50 µgml^{-1}), LPS (50 µgml^{-1}), Poly(I:C) (100 µgml^{-1}) and MHCS (50, 150 µgml^{-1}). Cells were subsequently harvested after 6, 12 or 24 h incubation at 27℃ with 5% CO_2, using 0.25x Trypsin-EDTA solution, washed 3 times with pre-warmed PBS and collected by centrifugation (800 g for 10 min at 4℃).

RNA was extracted from cell pellets using an RNeasy kit (Qiagen) and cDNA formed using the M-MLV RT kit (Invitrogen). Briefly, a mixture of 500 ng of RNA sample, 1 µl of 50 µM random hexamers, 1 µl 10 mM dNTPs and 4.5 µl of DEPC water were heated at 65℃ for 5 min before immediate cooling on ice. After a brief centrifugation, 4 µl of 5X First-Strand buffer, 2 µl of 0.1 M DTT and 1 µl of RNaseOUT™ recombinant ribonuclease inhibitor (40 units/ µl; Invitrogen) were added and mixed gently. The mixture was heated at 37℃ for 2 min and 1 µl (200 units) of M-MLV RT enzyme added before mixing thoroughly. The reverse transcriptase reactions were carried out after the samples were incubated at 25℃ for 10 min followed by 50 min at 37℃, then enzymes were heat inactivated by incubation at 70℃ for 15 min. Samples were diluted 1:10 (v/v) with DEPC treated water and stored at -20 ºC.

Carp specific primers were used to determine the expression levels of IL1β, TNFα1, TNFα2, iNOS and IFNα2 genes (Table 1); the ribosomal 40S gene was used as a housekeeping gene (Miest et al., 2012). The PCR reactions were carried out in 96 well PCR plates (Applied Biosystems, MicroAmp®). Briefly, 2 µl of cDNA were added to 10 µl SensiFAST, (Bioline, BIO-92020), 0.8 µl of 10 µM forward and reverse primers respectively

(reaction mixture final concentration equal to 400 nM) and made up to 20 µl with DEPC treated water (Invitrogen). The PCR plate was centrifuged (660 g, 4 min) (Boeco) prior to analysis. A cycling procedure was carried out with 1 cycle at 95°C for 2 min, 40 cycles at 95°C for 5 sec and 30 sec at 62°C. The melting curves of the PCR products were determined after each run between 60 and 95°C. The ΔΔCt method (Livak and Schmittgen, 2001) of targeted genes were normalised against the reference gene 40S, and the x-fold change calculated relative to the control group for each time point.

2.10 Statistical analysis

Statistical analyses were carried out using GraphPad Prism 5 and SPSS 21, with all data presented as the mean ± standard error. Data were tested for normality and equal distribution of variance. A one-way analysis of variance (ANOVA) and Tukey's post-hoc test were performed on the bioactivity data of MHCS with regard to cell proliferation, viability and ROS production. A two-way analysis of variance (ANOVA) and post-hoc Bonferroni's multiple comparisons test were used in the comparison experiments between semi-synthetic sulphated carbohydrates and different immunostimulants. Gene expression data were normalised using a Log10 transformation prior to a two-way ANOVA prior to conducting Bonferroni post-hoc test analyses. Significance was defined as $p \leq 0.05$.

3. Results

3.1 The effect of MHCS on cell viability & proliferation

The MTT cell proliferation assay demonstrated that the MHCS was not cytotoxic to the CLC line over the concentration range screened. Indeed, at concentrations of 1 and 2.5 μgml^{-1} MHCS promoted a significant increase ($p \leq 0.0011$, $p \leq 0.002$, respectively) in cell proliferation (Figure 1). The trypan blue exclusion assay confirmed this observation and supported the significant increase ($p \leq 0.026$) in cell growth after treatment to 2.5 μgml^{-1} of MHCS (Figure 2).

3.2 MHCS induced respiratory burst activity

Statistical analysis revealed a clear trend and significant increase in the respiratory burst activity of CLC cells after treatment with MHCS ($F = 68.668$, $p < 0.0001$). The concentration dependency effect of MHCS increased significantly ($p \leq 0.022$) at 25, ($p < 0.0001$) 50, 75, 150 μgml^{-1} and at the latter concentration, induced an increase in respiratory burst activity 4.5x greater than that of the control, 5 µl sterile water (Figure 3).

The respiratory burst activity in CLCs after treatment to methyl hydroxyethyl cellulose and MHCS confirmed that the modification of the polysaccharide by chemical sulphation conferred favourable bioactivity on the parental molecule (Figure 4). The levels of cell respiratory burst activity were significantly dependent upon the carbohydrate type ($F = 26.24$, $p < 0.0001$) and their concentrations ($F = 38.23$, $p < 0.0001$). The Bonferroni test revealed significant differences ($p < 0.001$) between the modified and precursor carbohydrates at concentrations between 25-250 μgml^{-1}, suggesting that the addition of sulphate moieties affects the biological activity of the cellulose ether. MHCS promoted the respiratory burst activity of CLC cells ($p < 0.0001$) in comparison to the control. The results further support the data presented in Figure 3.

Experiments on the respiratory burst activity of CLC line was extended to include MacroGard® and zymosan in comparison with the MHCS at concentrations in the range of 1-150 μgml^{-1} for 24 h. The assay demonstrated that the type of carbohydrate ($F = 834.8$, $p < 0.0001$), the concentration ($F = 211.4$, $p < 0.0001$) and the interaction of these two factors ($F = 172.3$, $p < 0.0001$) significantly influence the reactive oxygen species induced in CLC cells (Figure 5). The effect of MHCS was significant in comparison to MacroGard® and zymosan at concentrations of 25, 50, 100 and 150 μgml^{-1} (all at $p < 0.0001$). The chemically modified carbohydrate MHCS, stimulated CLC respiratory burst activity and presented a significant increase at concentrations ≥25 μgml^{-1} when compared against control (5 µl sterile water) ($p < 0.0001$).

3.3 Pathogen associated molecular patterns (PAMPs) and MHCS in pronephric cells

In order to determine whether MHCS treatment increased the immune response of pronephric cells under mock infection conditions, the level of reactive oxygen species (ROS) and effect on cell viability were determined post treatment to LPS and Poly(I:C) (Figure 6 and 7). ROS production in cells was significantly augmented ($p < 0.0001$) at 50 and 150 μgml^{-1} of MHCS irrespective of LPS treatment, when compared to the control (5 µl sterile water) (Figure 6 A). The treatment of pronephric cells with MHCS did not perturb cell viability at both concentrations either alone, or in combination with LPS (Figure 6 B). Treatment with MacroGard® at 50 and 150 μgml^{-1} alone, or with LPS, did not affect the

production of ROS. However, a significant decrease in cell viability was observed, when compared to control, for cells treated with MacroGard® at 50 μgml^{-1} with LPS ($p < 0.0001$), MacroGard® alone at 150 μgml^{-1} ($p = 0.024$) and MacroGard® at 150 μgml^{-1} with LPS ($p = 0.004$). Significant differences ($p = 0.015$) between MacroGard® at 50 μgml^{-1} and MacroGard® supplemented with LPS were also observed (Figure 6 B).

Treatment with Poly(I:C) induced a significant increase in ROS production alone ($p = 0.0002$) and in combination with either MacroGard® or MHCS ($p < 0.0001$) when compared to the control (5 μl sterile water) (Figure 7 A). The production of ROS in response to MacroGard® treatment at 50 and 150 μgml^{-1} had no effect, however when exposed with Poly(I:C), the ROS levels increased and were significantly different ($p = 0.005$, $p < 0.0001$ respectively) to MacroGard® alone. Both MHCS alone, and in the presence of Poly(I:C), induced highly significant increases in ROS production in comparison to the control ($p < 0.0001$; Figure 7 A). Interestingly, stimulation with MHCS at both concentrations induced a significant increase in ROS production of Poly(I:C) treated cells when compared to their respective concentrations of MHCS without Poly(I:C) and Poly(I:C) alone (Figure 7 A). The MTT assay shows no significant differences in cell proliferation, and hence viability in all treatment groups in comparison to the relevant control (Figure 7 B).

3.4 Immune gene expression in pronephric cells

The expression levels of the inflammatory cytokines IL1β and TNFα1 were increased significantly post treatment with MHCS (Figure 8). IL1β expression was up-regulated significantly ($p < 0.0001$) after 6 h post treatment at both MHCS concentrations and remained up-regulated after 12 ($p = 0.01$) and 24 h ($p = 0.03$) post treatment to MHCS at a concentration of 150 μgml^{-1}. While TNFα1 expression increased significantly after 6 h post treatment with both MHCS at 50 μgml^{-1} ($p = 0.015$) and 150 μgml^{-1} ($p = 0.0001$). Only MHCS at 150 μgml^{-1} affected the TNFα1 expression at 12 h ($p = 0.012$) and 24 h ($p = 0.008$) post treatment. However, the expression of TNFα2 was up-regulated significantly only at 6 h post treatment with MHCS at 50 μgml^{-1} ($p = 0.046$) and 150 μgml^{-1} ($p < 0.0001$).

MacroGard® induced significant IL1β expression after 6, 12 and 24 h post treatment ($p = 0.031$, 0.002, 0.021 respectively). Furthermore, a temporal response was observed in the expression of TNFα1 and TNFα2 at 6, 12 and 24 h post treatment with MacroGard®. The up-regulation was highly significant at all-time points ($p \leq 0.0001$) except for 6 h post treatment, where the expression of only TNFα1 was significant at $p = 0.002$. In contrast, elevated expression levels of IL1β, post treatment with LPS, were only induced significantly ($p = 0.007$) after 6 h post treatment. Furthermore, iNOS expression levels were increased significantly after 6 h treatment to MHCS, at concentrations of 50 and 150 μgml^{-1}, respectively ($p = 0.019$, $p < 0.0001$). LPS also induced significant ($p = 0.017$) iNOS expression at 6 h post treatment. In comparison, MacroGard® induced a late significant ($p < 0.0001$) iNOS expression at 12 and 24 h post treatment.

Poly(I:C) at 100 μgml^{-1} had no effect on either the inflammatory cytokines studied nor upon iNOS expression (Figure 8). However, the expression levels of IFNα2 were up-regulated significantly at all-time points studied after treatment to Poly(I:C) at 100 μgml^{-1} ($p < 0.0001$; Figure 8).

4. Discussion

The results show that a modified carbohydrate polymer such as MHCS can be generated with important biological activates and immunostimulatory effects in carp. Although, there are several methods to induce glucan modifications, sulphation has the strongest effects on biological function (Han et al., 2008). Previous studies in other non-fish systems have suggested that the sulphation process may alter the chemical and biological properties of glucans. For example, sulphated *Konjac glucomannan*, induces a high anti-HIV activity in the MT-4 cell line similar to the acquired immune deficiency syndrome (AIDS) drug (Bo et al., 2013). In another investigation, the presence of a sulphate group on the lentinan structure caused significant increases in antioxidant activity (Feng et al., 2010). Furthermore, rice bran β-glucan that was subjected to sulphation had a significant difference to the native oat glucan in molecular weight, solubility, viscosity and exhibited anticoagulant activity in rat blood (Chang et al., 2006).

The immune system recognises immunostimulants by the presence of pathogen recognition receptors (PRRs) that are present on the outer membrane of the immune cells. This recognition leads to activation of the immune cell and enhancement of their responses, which usually comprises an increase in their bactericidal activities, including the stimulation of phagocytosis, leucocyte migration and the production of cytokines (e.g. IL-1, TNFα), nitric oxide (NO) and reactive oxygen species (Sakai, 1999). In mammals, phagocytosis, believed to be the uptake mechanism of β-glucan, leads to their antimicrobial activity by the induction of reactive oxygen, and nitrogen species production and lytic enzymes in phagosomes (Goodridge et al., 2009). Several characteristic phagocytosis receptors on carp macrophage, including the complement receptor 3 (CR3), Scavenger Receptors (SRs) and C-type lectin receptor (CLR) superfamilies, and sensing receptors such as TLR2 (Petit and Wiegertjes, 2016) have been recognised as detecting β-glucan.

In addition, several studies have also highlighted the dose effects of β-glucans on cell cytotoxicity/ viability, for example in an investigation carried out on common carp, a significant increase in apoptosis occurred when pronephric cells were stimulated with β-glucans at concentrations 500 μgml^{-1} and higher for 6 h incubation (Miest and Hoole, 2015).

The MHCS was able to trigger several bioactive mechanisms i.e. cell viability, increase leucocyte number and respiratory burst activity. Interestingly, MHCS promoted a rapid increase in respiratory burst activity, which started at 25 μgml^{-1} concentrations and reached more than four and half times higher than the control at 150 μgml^{-1}. This linear dose/effect relationship is unusual for an immunostimulant because, often, the effect occurs at certain intermediate concentrations and disappears, or even becomes toxic at high concentrations (Kum and Sekkin, 2011). This steep increase in respiratory burst activity did not cause exhaustion to the immune cells as supported by the viability and cell count assays. This was a promising result, encouraging the debate whether the modification (sulphation) was the reason behind this biological effect. In a previous study it was shown that the soluble form of β (1-3)-glucan had some protective properties against infection in mice. The results of that study showed an increase in neutrophils in blood stream, enhancement in bone marrow proliferation and *in vitro* phagocytic activity to *E. coli* bacteria (Tzianabos, 2000).

The biological activities of modified carbohydrate MHCS were compared to the native source (cellulose ether) and different β-glucans. Interestingly, cellulose ether, zymosan and MacroGard® had no significant effects on CLCs line respiratory burst activity, while MHCS induced a significant increase in comparison to control and previous carbohydrates at a concentration equal to and higher than 25 μgml^{-1}. These rapid responses to MHCS carbohydrate by fish leucocytes might be due to carbohydrate-protein interactions. Sulphation

provides polysaccharides negative charges at the sulphate groups, which may be interacting with positive peptide sequence of proteins (Chang et al., 2006). This is in line with the many biological activities, which have been shown in heparin sulphation such as regulation of cellular growth and proliferation, cell adhesion, blood coagulation, cell surface binding of proteins, viral invasion, and tumour metastasis (Rabenstein, 2002).

Glucan solubility not only depends on the degree of polymerisation and branching, but also on chemical derivations including sulphation (Chang et al., 2006). The degree of substitution (DS) indicates the average number of sulphate groups attached to a glucose unit. Sodium rhodizonate assay was used to determine the DS of the sulphated derivative MHCS and was determined as 1.74 molesμg^{-1} of disaccharides. This demonstrates that sulphation was sufficient to induce important biological activities without causing detrimental side-effects e.g. cell death. When the degree of sulphation is high, there is an increased chance of undesirable anticoagulant activities for modified carbohydrates, e.g. highly sulphated carbohydrates such as dextran sulphate (degree of sulphation 5.25 molesμg^{-1} of disaccharides) has a high anticoagulant activity in human blood (Yoshida et al., 1995). Although the correlation between sulphation levels and anticoagulant potential is complex it should be noted that teleost fish coagulation system is fundamentally similar to that of mammals, in spite of the significant evolutionary distance between these groups (Tavares-Dias and Oliveira, 2009). However, blood coagulation time in fish is shorter in comparison to mammalian and depend on fish species (Wolf, 1959, Doolittle, 1962, Smit and Schoonbee, 1988).

It is possible that MHCS may form the basis of a new carbohydrate adjuvant in vaccine production. Therefore, before applying the MHCS to *in vivo* conditions, the effect of MHCS under mimicked infection conditions using PAMP immunostimulants were determined in carp pronephric cells. The results revealed that MHCS induced a significant increase in respiratory burst activity regardless of the LPS availability. LPS did not induce respiratory burst activity in pronephric cells when exposed alone, and had no additional effects on cells when exposed with MacroGard® or MHCS. This is despite the ability of LPS to stimulate the non-specific and specific immune responses in fish, and its recognitions by toll-like receptor 2 and 4 (TLR2, TLR4) in immune cells that induce a signalling cascade leading to the activation of NF-κB and the production of proinflammatory cytokines (Swain et al., 2008). However, responses to LPS can vary depending upon its source (Bich Hang et al., 2013), and its effects on macrophage respiratory burst activity appeared to be dose and incubation time dependent (Solem et al., 1995). Nayak et al. (2011) reported the high variability of the external polysaccharide region of LPS, and the differences in potency and spectrum of action of the lipid A components in many Gram-negative bacteria. This might be the explanation of the difference in the LPS effects on fish immunity, for example Watzke et al. (2007) noted the low sensitivity of zebrafish immune cells to LPS from *Escherichia coli* in comparison to *Edwardsiella tarda*. In addition, LPS from *Aeromonas hydrophila* stimulated carp (*Cyprinus carpio*) immune responses and enhanced fish protection against another aromonad *Aeromonas hydrophila* infection when exposed via intraperitoneal injection and bathing (Selvaraj et al., 2009).

Both MHCS and Poly(I:C) induced significant increases of the respiratory burst activity when exposed alone. Also, Poly(I:C) boosted the respiratory burst activity of both MacroGard® and MHCS treated cells. This might be due to the different uptake pathways of Poly(I:C), MacroGard® and MHCS. It is well established that Poly(I:C), which is used as a synthetic viral dsRNA analogue, induce IRF-3 (interferon regulatory factor-3) activation via the TLR3 (is an endosomal PRR of the innate immunity) and the synthesis of interferon-stimulated genes that restrict virus replication (Wang et al., 2009). While the main receptors

that are associated with glucans comprise C-type lectin receptor Dectin-1, complement receptor 3 (CR3), scavenger receptors (SRs), glycolipids or Carbohydrate Binding Module (CBM) (Legentil et al., 2015, Meena et al., 2012). Poly(I:C) recognition pathway was observed in pronephric cells of carp, where only Poly(I:C) induced significant up regulation of IFNα2 expression. IFN-alpha is a type I IFN that has a major role in the first line of defence against viruses. In mammals, the type I IFN antiviral effect is binding to the IFN-α/β-receptor, which triggers the JAK-STAT signal transduction pathway resulting in expression of Mx and other antiviral proteins (Robertsen, 2006). The difference in Poly(I:C) and MHCS uptake pathways might be the reason for the boosting of the immune cells responses more than when they are exposed alone.

MHCS induced the expression of pro-inflammatory cytokines (IL1β, TNFα1 and TNFα2), and inducible nitric oxide synthase (iNOS) 6h post treatment and the effect was dose dependant. Interestingly, the effect of MacroGard® was time dependant and increased with incubation time. MacroGard® time dependency was also observed by Miest and Hoole (2015) *in vitro*, where the pro-apoptotic effect was noted to be time and dose dependent only with concentrations of $\geq 500\ \mu g ml^{-1}$ causing apoptosis in carp pronephric leucocytes.

The above information is evidence of the ability of MHCS to be recognised by the innate immune system through pattern recognition receptors (PRRs) including Dectin-1 (β-glucan receptor (βGR), mannose receptor, complement receptors CR3, Toll-like receptors 2 and 6 (TLRs-2/6), scavenger receptors and lactosylceramide (Gantner et al., 2003, Herre et al., 2004, Chan et al., 2009, Kim et al., 2011). Glucans binding to the above receptors led to activation of several pathways and triggers several protection mechanisms i.e. phagocytosis, induction of pathogen killing activity, production of inflammatory cytokines and chemokines, and initiate the development of adaptive immunity (Gantner et al., 2003). β-glucan is involved in the enhancement of mononuclear cells and neutrophil anti-microbial activity, leading to improve macrophage activity and the proliferation of both monocytes and macrophages, and the production of proinflammatory molecules such as complement components, interleukin (IL)-1α/β, TNF-α, IL-2, interferon (IFN)-γ, IL-4 and IL-10 (Chan et al., 2009, Kim et al., 2011, Li et al., 2013). Different glucans associated with different or similar receptors on immune cells do not induce the immune response equally. For example, the scavenger receptors are non-opsonic receptors that have low affinity to attach to anionic β-glucans, which have been sulphated chemically or originate from natural sources (algae) (Meena et al., 2012). While Dectin-1 receptor has been identified as a major receptor for β-glucans on mammalian leucocytes (Herre et al., 2004). The solubility and smaller molecule size of MHCS might result in a rapid engulfment and lysis by the immune cells in comparison to MacroGard. The adjuvant size determines the ability and speed to diffuse inside tissues and reach the target. At sizes less than 40 nm, transmission is more rapid than the large size adjuvant of 100 nm that occurs in polysaccharides, which are transported from the injection site by dendritic cells to the immune organs (Smith et al., 2013). The new smaller size MHCS thus has potential to have an increased immunostimulant affect in fish when compared to the larger size carbohydrates which are currently in use e.g. β-glucan. The utilisation of this new carbohydrate in an *in vivo* system is in progress.

5. General conclusion of the study

The present study contributes to the development of the use of carbohydrates as immunostimulants in fish. This represents the first attempt to combine synthetic biochemical approaches with carbohydrate design to produce a novel carbohydrate that modulates the immune system at the cellular and molecular level. The MHCS exhibited a range of bioactive

properties such as the non-cytotoxic effect, and production ROS in immune cells. These bioactivities were associated with the sulphate group in this carbohydrate structure. Also, there is the potential to use this modified carbohydrate as an adjuvant in vaccines as it was able to increase the immune response in mimic infection conditions and up-regulate the expression of inflammatory cytokines genes.

Therefore, the next step is to trial MHCS and *in vivo* conditions and evaluate the adjuvant potency in vaccines against important diseases in aquaculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at:

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Figure legends

Figure 1: CLC line viability exposed to serial dilution of modified carbohydrates. Cells at density (2×10^4 cells per well) stimulated with zymosan = Z, MacroGard® = M at 50 µgml^{-1} and range of modified carbohydrates concentrations 1 - 150 µgml^{-1} for 24 h. Statistical analyses were performed by one-way ANOVA ($p \leq 0.05$) and the significant differences between treatments in comparison to control performed with *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$. Data represent mean ± SEM of 6 well replicates.

Figure 2: CLC line count utilised by trypan blue viability assay. Cells were exposed to serial dilution of modified carbohydrates MHCS (1-150 µgml^{-1}) and MacroGard® at 50 µgml^{-1} concentration for 24 h. Statistic comparison was performed by one-way ANOVA and the significant differences between treatments in comparison to control performed with *: $p \leq 0.05$. Data represent mean ± SEM of 3 well replicates of 24 well plates.

Figure 3: Dose dependency effect of modified carbohydrates on CLCs respiratory burst level. Cell were seeded at (2×10^4 cells per well) and stimulated with zymosan at 50 µgml^{-1} = Z, MacroGard® at 50 µgml^{-1} = M and range of modified carbohydrates at concentration between 1-150 µgml^{-1} for 24 h incubation. Statistical analysis one-way ANOVA and Tukey's *post hoc* analysis were used and the differences between concentrations in comparable to control (non-treated cells) performed with *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.0001$. Data represent mean ± SEM of six well replicates.

Figure 4: CLCs dose responses to sulphated and non-sulphated MHCS determined by NBT assay. The cells were distributed at (2×10^4 cells per well) stimulated with a range of cellulose ether and sulphated cellulose ether (MHCS) at concentrations between 2.5-250 µgml^{-1} for 24 h. Statistic comparison was performed using two-way ANOVA and Bonferroni *post hoc* test at $p \leq 0.05$ and the significant differences between concentrations in comparison to control presented with ***: $p < 0.0001$, also the comparison between the two treatments at each concentration presented with ΔΔΔ: $p < 0.0001$. Data represented the mean ± SEM of 6 well replicates.

Figure 5: Comparison of distinct β-glucan sources and MHCS carbohydrate on CLCs phagocytic activity. Cells phagocytic activity was measured by NBT assay after cells were distributed at (2×10^4 cells per well) and simulated with either MacroGard®, zymosan or MHCS at concentrations 1-150 µgml^{-1} for 24 h. Statistical analysis was performed by two-way ANOVA and Bonferroni *post hoc* test ($p \leq 0.05$) and the significant differences between MHCS concentrations in comparison to matched control performed with ***: $p \leq 0.0001$. Data represent mean ± SEM of 6 well replicates.

Figure 6: Respiratory burst production (**A**) and viability (**B**) in carp leucocytes after treatment to LPS (50 µgml^{-1}), MacroGard® (50 or 150 µgml^{-1}) and MHCS (50 or 150 µgml^{-1}) both exposed individually and in combination with LPS. Statistical analysis one-way ANOVA and Tukey's *post hoc* analysis were used and the differences between concentrations in comparable to control (non-treated cells) and the LPS availability was performed with N.S: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.0001$. Bars represent the mean of 3 wells from 5 fish ± SEM.

Figure 7: Respiratory burst production (**A**) and viability (**B**) in carp leucocytes after treatment to Poly(I:C) (100 $\mu g ml^{-1}$), MacroGard® (50 or 150 $\mu g ml^{-1}$) and MHCS (50 or 150 $\mu g ml^{-1}$) both exposed individually and in combination with Poly(I:C). Statistical analysis one-way ANOVA and Tukey's *post hoc* analysis were used and the differences between concentrations in comparable to control (non-treated cells) and the Poly(I:C) availability was performed with N.S = not significant, *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.0001$. Bars represent the mean of 3 wells from 5 fish ± SEM.

Figure 8: Effect of different immunostimulant and exposure time on immune gene expression in carp pronephric leucocytes. Bars represent mean of relative expression normalized to housekeeping gene 40s ± SEM of three fishes. Two ways ANOVA followed by Bonferroni *post hoc* analysis used to compare each treatment to the time matched control *: $p \leq 0.05$; **: $p \leq 0.01$ and ***: $p \leq 0.001$.

833 **Tables**

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835 Table1: List of used qPCR primers

Function	Gene name	Primers sequences	Gene bank accession numbers	References
House keeping	40S	FW: 5′ CCGTGGGTGACATCGTTACA 3′	AB012087	(Huttenhuis et al., 2006)
		RV: 5′ TCAGGACATTGAACCTCACTGTCT 3′		
Nitric oxide production	iNOS	FW: 5′ AACAGGTCTGAAAGGGAATCCA 3′	AJ242906	(Huttenhuis et al., 2006)
		RV: 5′ CATTATCTCTCATGTCCAGAGTCTCTTCT 3′		
Pro-inflammatory cytokines	IL1β	FW: 5′ AAGGAGGCCAGTGGCTCTGT 3′	AJ245635	(Falco et al., 2012)
		RV: 5′ CCTGAAGAAGAGGAGGCTGTCA 3′		
	TNFα1	FW: 5′ GAGCTTCACGAGGACTAATAGACAGT 3′	AJ311800.2	(Falco et al., 2012)
		RV: 5′ CTGCGGTAAGGGCAGCAATC 3′		
	TNFα2	FW: 5′ CGGCACGAGGAGAAACCGAGC 3′	AJ311801.2	(Falco et al., 2012)
		RV: 5′ CATCGTTGTGTCTGTTAGTAAGTTC 3′		
Anti-viral cytokines	IFNα2	FW: 5′ GATGAAGGTGCCATTTCCAAG 3′	AB376667	(Adamek et al., 2014)
		RV: 5′ CACTGTCGTTAGGTTCCATTGCTC3′		

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839 **Figure 1**
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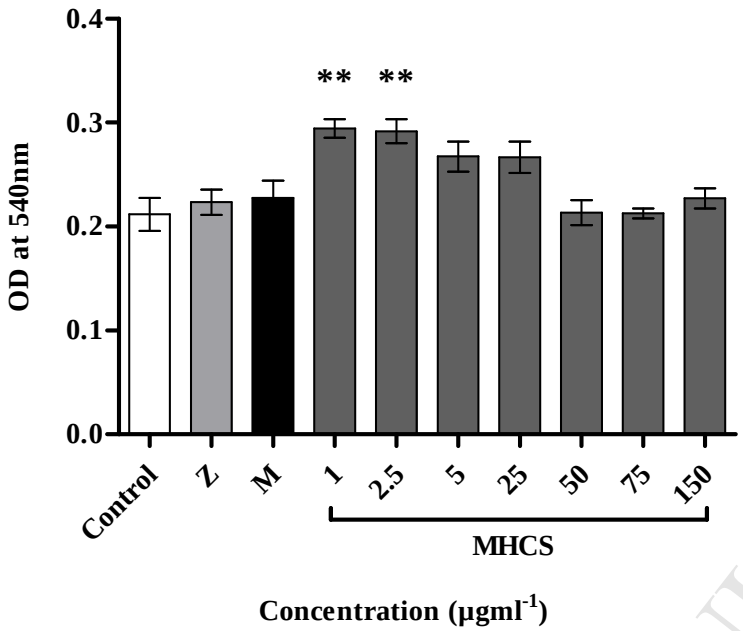

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843 **Figure 2**
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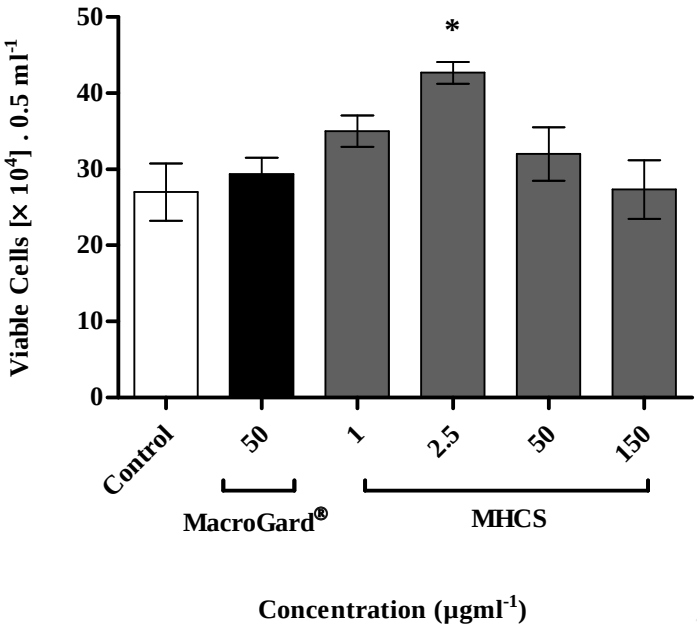

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848 **Figure 3**
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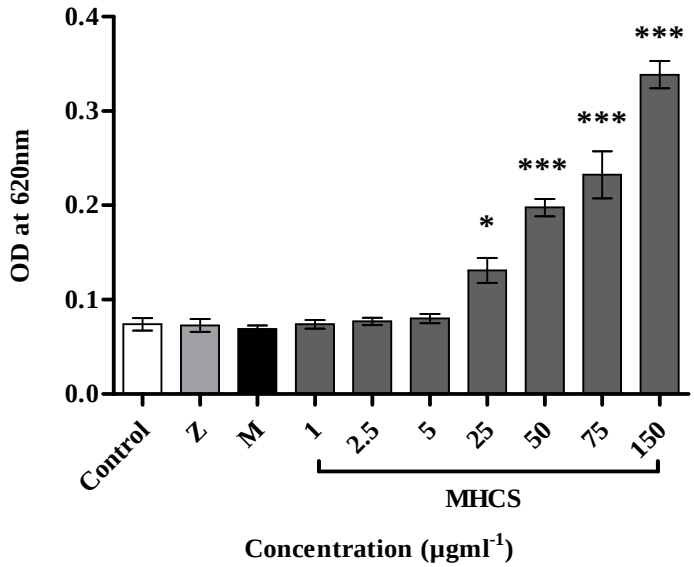

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853 **Figure 4**
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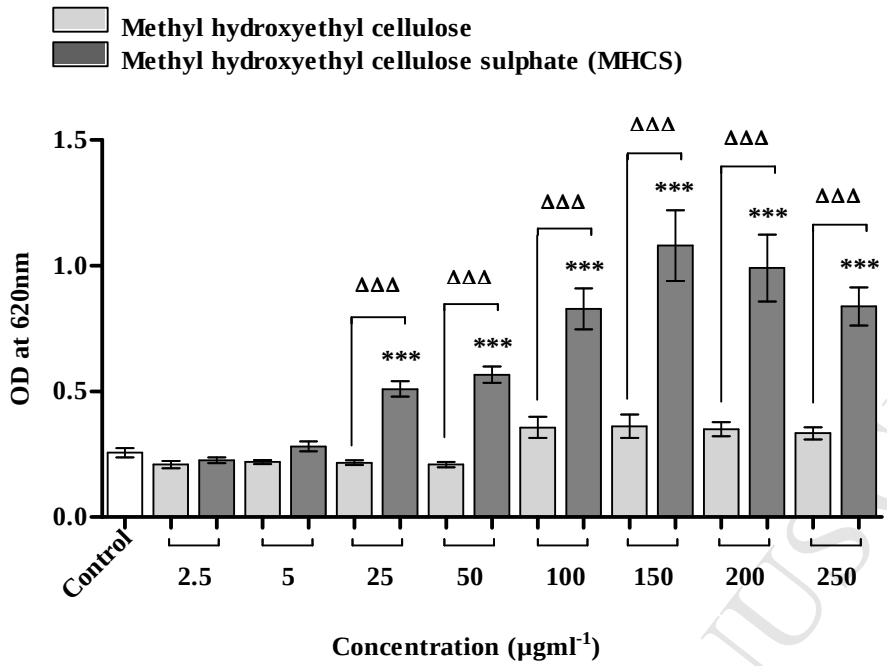

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857 **Figure 5**
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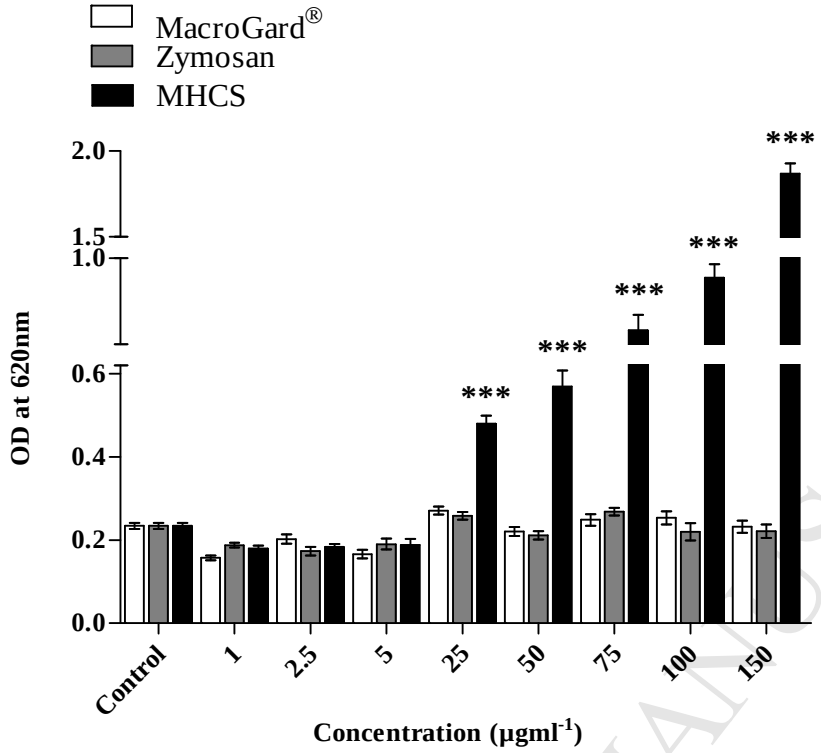

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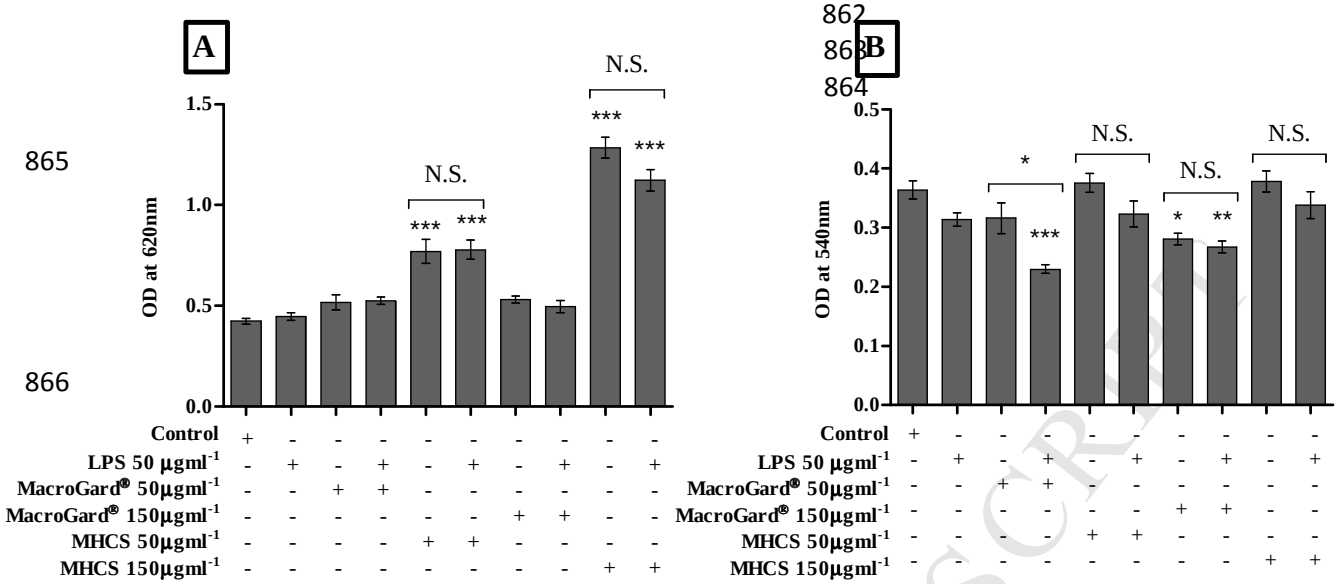
Figure 6



872 **Figure 7**

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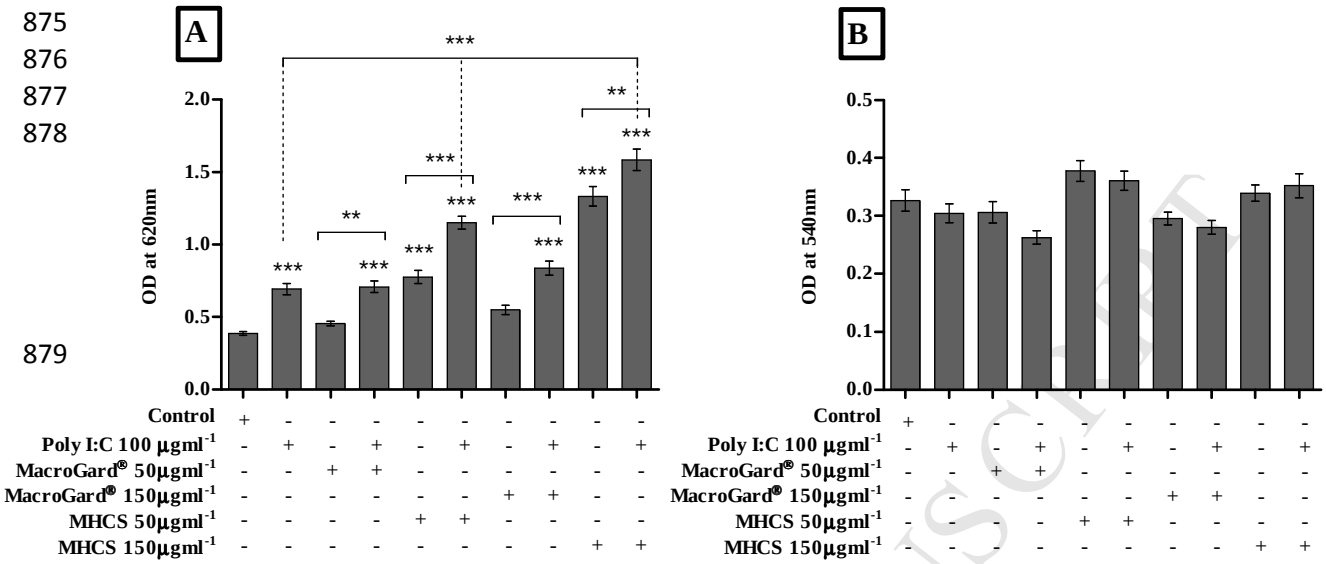

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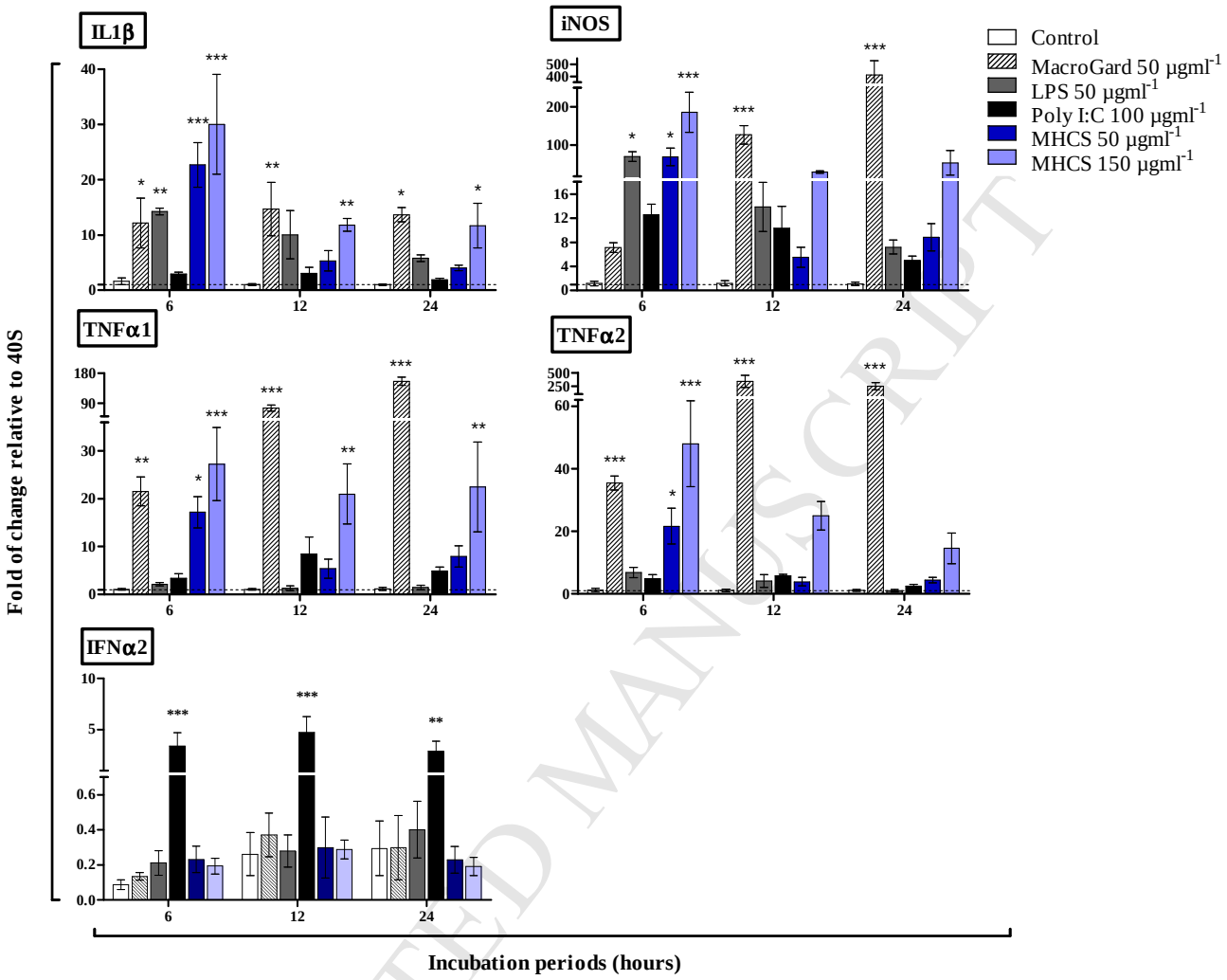
Figure 8



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In vitro investigations on the effects of semi-synthetic, sulphated carbohydrates on the immune status of common carp (Cyprinus carpio).

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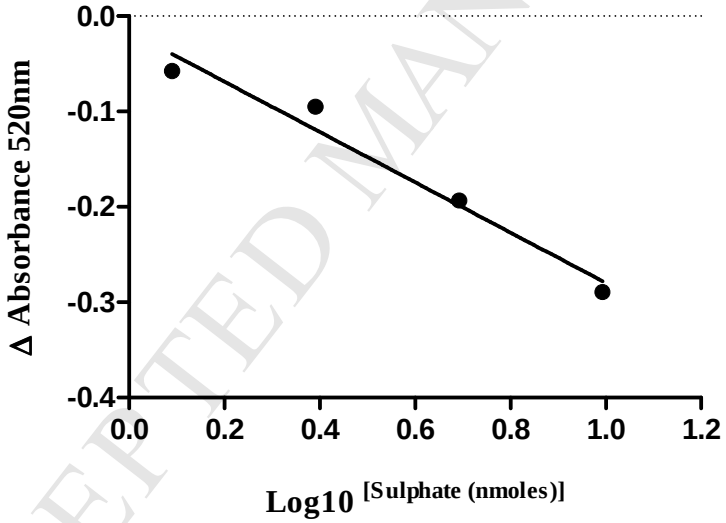
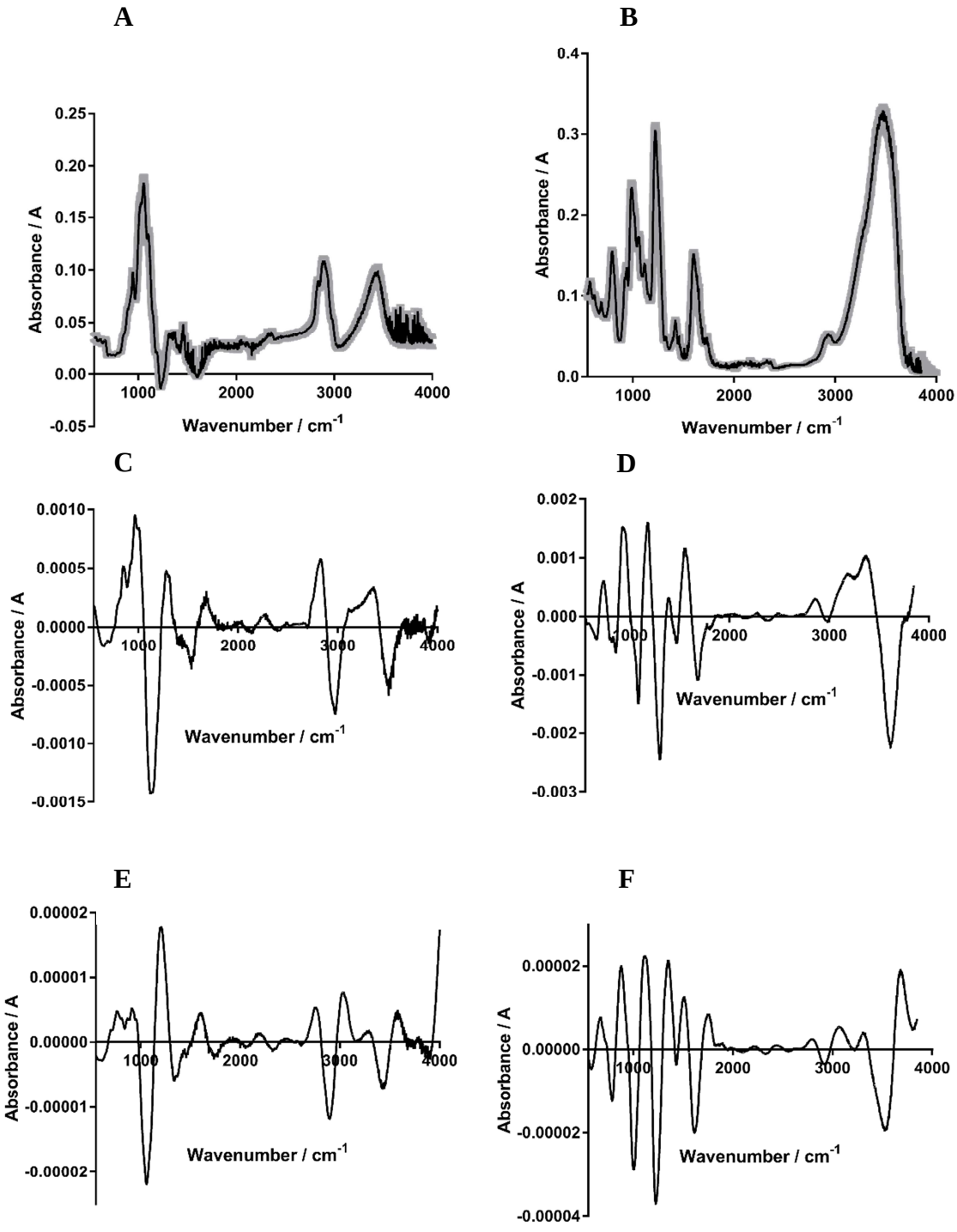
SUPPLEMENTARY DATA



Supplementary Figure 1: Standard curve for dextran sulphate using the sulphate determination assay, employing sodium rhodizonate, as described by Terho and Hartiala, Anal. Biochem. 1971, 41(2):471-6. $y = 0.2637x - 0.0158$; $R^2 = 0.9674$.

The overall sulphation level for methyl hydroxyethyl cellulose sulphate was determined by the method of Terho and Hartiala (Anal. Biochem. 1971, 41(2):471-6). Briefly, dextran sulphate, of a predetermined degree of sulphation, was hydrolysed in 1 M HCl (100°C for 2 hours) prior to lyophilisation. The dry product was reconstituted in sterile water (1 $mgml^{-1}$) and serial dilutions performed (calibration curve) before the addition of 0.1 M CH_3CO_2H, 50 uM $BaCl_2$, 0.8 mM $NaHCO_3$, 0.14 mM sodium rhodizonate and 3.4 mM L-(+)-ascorbic acid. The solution was incubated for 10 min at 20°C (in darkness) to allow colour to develop. The absorbance of the solution was ascertained at λ_{abs}= 520 nm. The assay

was repeated for methyl hydroxyethyl cellulose sulphate and the mass of sulphate per gram of polysaccharide calculated from the dextran sulphate calibrant.



Supplementary Figure 2: FTIR-ATR spectra for both the unsulphated (A) and sulphated (B) methyl hydroxyethyl cellulose polysaccharides within the 400-4000 cm^{-1} spectral region. First ((C) and (D)) and second derivative ((E) and (F)) curves are shown, respectively.

Attenuated total reflectance FTIR spectra were recorded for the MHCS carbohydrate and the precursor using a Nicolet iS5 IR-TF (Thermo Fisher) spectrometer scanning in the 4000–400 cm-1 region with a spectral resolution of 2 cm^{-1} over 32 scans. A background air spectrum was obtained and subtracted from all spectra. All carbohydrate spectra were recorded using ThermoFisher Omnics software. First and second derivatives of all spectral data for the precursor and modified polysaccharide were plotted and overlaid using Prism software (GraphPad Software, Inc.).